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Prevalence and genotypes of *Giardia duodenalis* in post-weaned dairy calves

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Abstract

To determine the prevalence of *Giardia* genotypes in post-weaned dairy calves, fecal specimens were collected from 3 to 11-month-old dairy calves per farm on two farms in Vermont, New York, Pennsylvania, Maryland, Virginia, North Carolina, and Florida. Specimens cleaned of fecal debris and concentrated using CsCl density gradient centrifugation were stained and examined by immunofluorescence microscopy and also subjected to PCR and DNA sequence analysis. Overall, PCR provided more sensitive detection than IFA. Prevalence of *Giardia* infection, as detected by PCR ranged from 20% on NC-2 to 81% on VT-2, with an overall prevalence of 52% (237 positive samples out of 456 total). DNA sequence analysis of the 16S rRNA gene revealed 87% of the 237 *Giardia* isolates were Assemblage E, and 13% were Assemblage A although the prevalence of these genotypes varied greatly from farm to farm, with five farms having no Assemblage A *Giardia*. Therefore, Assemblage E was present in 45% of all animals tested and Assemblage A was present in 7% of the animals. Thus, while many of the calves were infected with a genotype that is not known to be infectious for humans, post-weaned calves on nine of 14 farms did harbor Assemblage A *Giardia*. Therefore calves should be considered as a potential source of human infectious cysts in the environment, with some farms representing a much higher risk than others.

Keywords: Calves; Genotype; *Giardia* spp.; PCR; Prevalence

1. Introduction

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*) is a commonly identified intestinal parasite of mammals, including humans. Giardiasis has been the most commonly diagnosed disease associated with drinking water in United States (Lee et al., 2002), however, the potential sources of cysts infectious for humans are not well known. Identification of animal sources of *Giardia* is complicated by the fact that although genetic and biological differences exist within the *G. duodenalis* complex, subtypes are morphologically indistinguishable (Thompson et al., 2000). Thus, it appears that not all organisms in the *G. duodenalis* grouping have the same

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biological potential to infect humans. Molecular analysis has proven useful for identifying genotypes or assemblages, and characterizing their host ranges (Monis et al., 1999, 2003). Assemblages A and B have the widest host ranges, including humans and a number of other mammals; Assemblages C and D have been reported in dogs, Assemblage E in hoofed livestock, Assemblage F in cats, and Assemblage G in rats (Monis et al., 2003).

The prevalence of *Giardia* infection in cattle varies markedly. While many point prevalence studies of cattle report a significant percentage of *Giardia*-infected animals, cumulative prevalence often reaches 100% (Olson et al., 1997a,b; Ruest et al., 1998; O'Handley et al., 1999; Ralston et al., 2003). Despite the abundant prevalence information, there is little information on the genotypes that are present in infected cattle in the United States. Assemblage E is the predominant genotype found in Canadian cattle, but a small percentage of Assemblage A has also been found (O'Handley et al., 2000; Appelbee et al., 2003). Analysis of three bovine specimens from New York indicated the presence of Assemblage A in cattle as well (van Keulen et al., 2002).

In a multi-state prevalence study for *Giardia* in the eastern United States, pre-weaned calves (<2 months of age) were found infected with both Assemblage E and Assemblage A. On average, Assemblage A represented 15% of the *Giardia* that was isolated, ranging from a low of 0% of the isolates on seven of 14 farms to a high of 45% of the isolates on a farm in New York (Trout et al., 2004). Thus, on certain farms, a significant percentage of the *Giardia* isolated from pre-weaned calves had zoonotic potential. However, there is currently little information available on *Giardia* genotypes in older dairy calves.

The current study was conducted to assess the prevalence of *Giardia* in post-weaned dairy calves in the eastern U.S. and to identify the genotypes that were present.

2. Materials and methods

2.1. Farms

Two commercial dairy farms in Vermont, New York, Pennsylvania, Maryland, Virginia, North Carolina, and Florida were selected based on availability of a minimum of 15 calves of the appropriate age for sampling.

2.2. Calves

Calves (primarily, but not exclusively, females) between the ages of 3 and 11 months of age were randomly selected for sampling on each farm. The number of animals from which useful specimens were obtained ranged from 21 to 49 per farm (Table 1).

2.3. Fecal sample collection and processing

Fecal samples were collected and processed as described in Trout et al. (2004). Briefly, feces were collected from each calf into plastic screw cap specimen cups processed within 4 days of collection. Fifteen grams of feces were mixed with water and passed through a 45 μm screen. The samples were then subjected to density gradient centrifugation using CsCl (1.4 g/ml). Following centrifugation, the top 4 ml of supernatant were aspirated from each sample and transferred into a 15 ml tube, and samples were washed twice with dH_2O; the final pellet was suspended in 500 μl of dH_2O. Portions of this 500 μl suspension were used for immunofluorescence analysis or molecular analysis as described below. Eight samples from which direct smears were prepared could not be subjected to molecular analysis due to insufficient sample material for DNA extraction.

2.4. Immunofluorescent analysis

From each fecal suspension, a 100 μl aliquot was transferred to a microcentrifuge tube and washed once with dH_2O. The pellet was suspended in 25 μl of premixed MerIFluor reagents (Meridian Diagnostics, Cincinnati, Ohio). Premixed MerIFluor reagents were prepared as follows: combine MerIFluor test reagent and counter stain, add 3 ml sterile PBS and mix well. Two microlitre of the fecal/stain suspension was transferred to one well of a 3-well slide (Cel-Line, HTC, Portsmouth, NH); a coverslip was placed on the slide and slides were examined at 400× using a Zeiss Axioskop microscope equipped with epifluorescence

Table 1
Farm designations, number of calves sampled on each farm, and prevalence of *Giardia duodenalis* and genotypes in post-weaned dairy calves on 14 farms in seven East Coast states

State	Farm	Number of animals sampled	Number Positive by IFA	Number of samples analyzed by PCR	Number Positive by PCR	Assemblage (percent of PCR positive isolates)	
						A	E
Vermont	VT-1	27	0 (0%)	27	19 (70%)	0	100
	VT-2	26	14 (53%)	26	21 (81%)	24	76
New York	NY-1	32	6 (19%)	28[a]	20 (71%)	0	100
	NY-2	30	6 (20%)	26[a]	19 (73%)	5	95
Pennsylvania	PA-1	49	18 (37%)	49	19 (39%)	0	100
	PA-3	33	15 (45%)	33	19 (58%)	26	74
Maryland	MD-1	35	6 (17%)	35	9 (26%)	67	33
	MD-2	26	15 (58%)	26	15 (58%)	0	100
Virginia	VA-1	21	3 (14%)	21	14 (67%)	29	71
	VA-2	40	5 (13%)	40	13 (33%)	0	100
North Carolina	NC-1	38	5 (13%)	38	18 (47%)	17	83
	NC-2	35	7 (20%)	35	7 (20%)	14	86
Florida	FL-1	43	24 (56%)	43	34 (79%)	12	88
	FL-2	29	19 (66%)	29	10 (34%)	10	90
Total		464	143 (31%)	456	237 (52%)	13	87

[a] Difference from number of animals sampled reflects insufficient fecal material for DNA extraction.

and an FITC-Texas Red dual wavelength filter. Slides with direct fecal smears were stained by covering the sample with 100 μl of pre-mixed MerIFluor reagent and incubating at room temperature for 30 min. Each slide was rinsed with dH_2O, coverslipped and examined as described above.

2.5. DNA extraction

Total DNA was extracted from each CsCl-cleaned fecal sample using a DNeasyTissue Kit (Qiagen, Valencia, California) with a slightly modified protocol. The protocol, described below, utilized reagents provided by the manufacturer. A total of 50 μl of processed feces were suspended in 180 μl of ATL buffer and thoroughly mixed by vortexing. To this suspension, 20 μl of Proteinase K (20 mg/ml) was added, and the sample was thoroughly mixed. Following an overnight incubation of the mixture at 55 C, 200 μl of AL buffer was added. The remaining protocol followed manufacturer's instructions with one exception. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μl of AE buffer.

2.6. Polymerase chain reaction and DNA sequence analysis

A fragment of the ssrRNA (~292 bp) gene was amplified by PCR as previously described (Hopkins et al., 1997). PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

PCR products were purified using EXO-SAP enzyme (USB Corporation, Cleveland, Ohio). Purified products were sequenced with the same PCR primers used for the original amplification in 10 μl reactions, Big Dye™ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, California). Each sample was sequenced in both directions. Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Inc., Madison, Wisconsin). All of the PCR positive samples were sequenced.

3. Results

The number and location of calves infected with *Giardia* as determined by IFA and PCR are shown in

Table 1. The differences in total numbers of samples analyzed between these two methods reflects the fact that occasionally, insufficient fecal material was available for DNA extraction. Of 464 calves on 14 farms examined by IFA, 143 (31%) were *Giardia* positive, and of 456 samples examined by PCR, 237 (52%) were *Giardia* positive, thus PCR provided more sensitive detection. The prevalence of *Giardia* infection varied considerably across farms, with the lowest prevalence (20%, by PCR) on NC-2 and the highest prevalence (81%, by PCR) on VT-2. Overall, on eight of the 14 farms greater than 50% of the calves were found to be PCR positive for *Giardia*.

The percentages of *G. duodenalis* genotypes found on the farms are presented in Table 1. Two genotypes were identified: Assemblage E, which has been reported to infect only hoofed-livestock, and Assemblage A, which is infectious for humans and a number of other mammals. Across all farms, 13% of the *Giardia* positive animals were infected with Assemblage A, while 87% were infected with Assemblage E. Assemblage E was found on all farms sampled, while Assemblage A was found on nine of 14 farms: two of four farms in the northeast, three of six farms in the mid-Atlantic, and four of four farms in the south. On farms where Assemblage A was present, it represented 20% of the *Giardia* positive calves; the lowest percentage was on FL-2 (10%) and the highest percentage was on MD-1 (67%).

For 10 farms (NY-1, NY-2, PA-1, MD-1, MD-2, VA-2, NC-1, NC-2, FL-1, FL-2) exact dates of birth were provided and *Giardia* prevalence was determined by months of age. Fig. 1 shows the prevalence of total *Giardia* as well as that of Assemblage A and Assemblage E; the figure includes data on calves 2 months of age and younger from our previous study

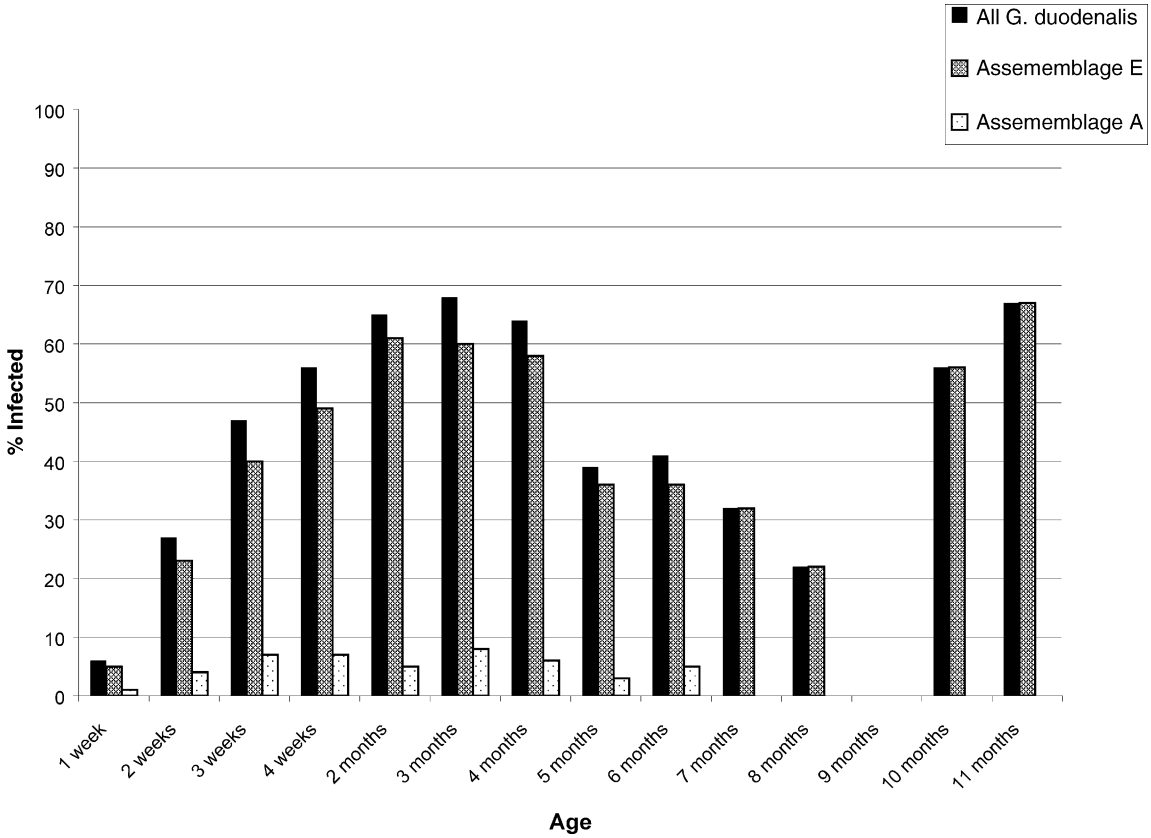

Fig. 1. Prevalence *Giardia duodenalis* by age in pre- and post-weaned dairy calves averaged over 10 farms in seven East Coast states. Data for calves 2 months of age and younger is adapted from Trout et al. (2004).

(Trout et al., 2004). Prevalence of total *Giardia* and that of Assemblage E was highest at 3 months of age and then declined through 8 months of age. The percentage of Assemblage A was relatively constant between 2 weeks and 6 months of age. The data for 10 and 11 months of age represent only nine and six calves in each age group, respectively, on the same farm, and thus likely do not provide an accurate assessment of the prevalence in these age groups.

4. Discussion

Giardia infection has been reported frequently in calves. Point prevalence studies often report widely varying levels of infection, while the cumulative prevalence for a given farm often reaches 100% (Xiao and Herd, 1994; O'Handley et al., 1999). Cyst excretion, however, can be intermittent (Buret et al., 1990), especially near the end of the infection. Because only one fecal sample was collected from each animal in the present study, it is likely that the point prevalence data presented herein underestimates the actual numbers of infected animals. *G. duodenalis* infections were detected in post-weaned dairy calves on all 14 farms examined, with prevalence by PCR ranging from 20 to 81%. Previous point prevalence studies have also reported a wide range in the number of *Giardia* infected animals (Xiao, 1994; Xiao and Herd, 1994; Olson et al., 1997a,b; O'Handley et al., 1999, 2000). Other studies (O'Handley et al., 1999; Huetink et al., 2001) indicated that the prevalence of *Giardia* infection reached the highest levels at 4–5 months of age. However, in the current study, the prevalence of infection reached 68% in 3-month-old calves and then declined through 8 months. The increased prevalence seen at 10 and 11 months represented a small number of animals on only one farm, and thus likely does not provide an accurate assessment of the true prevalence at these ages.

The prevalence of *Giardia* genotypes was determined by DNA sequence analysis of the 16S rRNA gene for every PCR positive sample. Thus, sequence data were obtained for 237 samples. Assemblage A *Giardia* (the most common human genotype) was detected at varying levels in calves on nine of the 14 farms. Assemblage E *Giardia*, with a host range limited to hoofed-livestock, was detected in calves on all farms with five farms having exclusively Assemblage E. On farms where Assemblage A was detected, the prevalence ranged from 10 (FL-2) to 67% (MD-1) of the isolates. Thus, while Assemblage A represented 13% of the isolates in the study as a whole, averaging only the nine farms where Assemblage A was detected, this genotype represented 20% of the positive isolates. Previous studies have reported the presence of Assemblage A in both dairy and beef calves (O'Handley et al., 2000; Appelbee et al., 2003; Trout et al., 2004). One of these studies (Trout et al., 2004) conducted by our laboratory sampled pre-weaned calves on 13 of the 14 farms sampled in the current study; the PA-2 farm did not have sufficient calf numbers and was replaced with PA-3. In that study of pre-weaned calves, Assemblage A *Giardia* was detected on seven of 14 farms, and represented 15% of the total *Giardia* isolates (or 6% of all the animals sampled). Thus, although the average prevalence of Assemblage A was similar in post-weaned animals, it was detected on two additional farms. A similar study of *Cryptosporidium* species and genotypes (Santín et al., 2004), reported the pre-weaned calves were the primary source of the zoonotic species, *C. parvum*, while post-weaned calves were primarily infected with species and genotypes that were not infectious for humans. This change does not appear to occur with *Giardia*, in that the percentage of Assemblage A appears to be relatively constant, when comparing pre- versus post-weaned animals. When data are grouped by age averaged over all of the farms, the percentage of Assemblage A appears relatively constant with about 6% of pre-weaned animals (Trout et al., 2004) and 7% of all post-weaned animals infected with this genotype. However, this averaging obscures the changes that are noted on individual farms between pre- and post-weaned calves, and thus do not provide an accurate assessment of the variation within an individual farm of the prevalence of the two Assemblages.

In our previous study (Trout et al., 2004), four of four farms in the Northeastern states had cattle positive for Assemblage A, representing 35% of the *Giardia* isolates. Cattle on three of six farms in the Mid-Atlantic states, were positive for Assemblage A, an average of 12% of the *Giardia* isolates. In the Southeastern states none of the *Giardia* isolates

obtained from cattle on 4 farms was Assemblage A. However, in the current study of post-weaned calves, no similar pattern was observed. The current data showed two of four farms in the Northeast were positive for Assemblage A, an average of 8% of the isolates, and again three of six farms in the Mid-Atlantic were positive for Assemblage A, an average of 17% of the isolates. In the Southeast, however, all four farms were now positive for Assemblage A, an average of 13% of the isolates. Thus, there does not appear to be any geographical effect on the distribution of this genotype. It is interesting, however that on four southeastern farms, where no Assemblage A was previously detected, this genotype now represented 13% of the *Giardia* isolates. The appearance of Assemblage A where none was detected previously, suggests its possible introduction by some factor related to variation in management practices used for pre- versus post-weaned animals. The decrease in Assemblage A seen in the Northeast, could be more simply explained due to the animals being exposed at an earlier age and subsequently clearing the infections. There are no data available to determine if the duration of cyst shedding for Assemblage A and Assemblage E in cattle are similar, but it is possible that these genotypes have different patent periods.

In comparing genotype with age on the 10 farms for which dates of birth could be obtained, it appears that the peak in prevalence seen at 3 months of age is due to an increase the number of animals infected with Assemblage E, as Assemblage A remained at relatively constant levels. It is not clear if Assemblage A is no longer present in calves over 6 months of age as suggested by the data presented in Fig. 1. A study is currently underway to examine 12–24 month old animals, and this should provide additional information.

The present study provides genotype data for all *Giardia* positive cattle on a small number of farms over a wide geographical area. The presence of Assemblage A on nine of 14 of the farms and its high prevalence on several farms indicate that post-weaned calves can be a source of zoonotic *G. duodenalis* cysts and that the distribution and prevalence are irregular and unpredictable. This variation complicates our ability to estimate the risk cattle as a reservoir of *Giardia* with the potential to infect humans.

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